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ORIGINAL PAPER

Long-term gus expression from *Gladiolus* callus lines containing either a *bar-uidA* fusion gene or *bar* and *uidA* delivered on separate plasmids

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Abstract Gus expression was determined for 19 lines of embryogenic *Gladiolus* callus that contained the 35S-*bar-uidA-nos* fusion gene and for 21 callus lines that had been cobombarded with the 35S-*bar-nos* and 35S-*uidA-nos* plasmid DNAs. These lines were selected for analysis because they grew vigorously on Murashige and Skoog's medium supplemented with 6 mg l^{-1} phosphinothricin. All 19 lines that contained the 35S-*bar-uidA-nos* fusion gene expressed gus compared to only 15 (71%) of the lines that had been cobombarded as determined by enzyme assay. The level of gus expression was significantly higher the first year for 12 callus lines containing the *bar-uidA* fusion gene as compared to 2 years later in culture. Southern hybridization confirmed integration of the *uidA* gene in all callus lines that had been bombarded with the 35S-*bar-uidA-nos* fusion gene. Two of the callus lines that had been cobombarded lacked the *uidA* gene, and another cobombarded line that did not express gus contained a truncated *uidA* gene. Two callus lines resulting from cobombardment showed gus expression in only a few cells indicating that gus expression was not completely silenced in these lines. Gus expression could not be reversed using 5-azacytidine in these two low-expressing lines, and Southern hybridization supported that methylation of the genomic DNA had not occurred. Average levels of gus expression were significantly higher, 8.9× , in cells with the 35S-*bar-uidA-nos* fusion gene compared to the cobombarded callus lines indicating the advantage of using a *bar-uidA* fusion gene for obtaining higher levels of gus expression in *Gladiolus*.

Keywords Bulb crops · Floral monocot · Reporter gene

Abbreviations

CaMV 35S	Cauliflower mosaic virus 35S promoter
2,4-D	2, 4-Dichlorophenoxyacetic acid
MS	Murashige and Skoog's medium

Introduction

The *uidA* gene that encodes *β*-glucuronidase (gus) (Jefferson and Wilson 1991) remains an important reporter gene. Previously we had reported that the bifunctional 35S-*uidA-bar-nos* fusion construct resulted in the isolation of 3× more cell lines that expressed gus than cobombardment with plasmids, each containing either the *bar* or *uidA* gene under control of the CaMV 35S promoter (Kamo et al. 2000). The short and long-term levels of gus expression conferred using the fusion gene or cobombardment were not reported. In plants, other fusion genes used include the *GOS5* gene fused to the *uidA* gene, and gus expression was demonstrated in three monocot species (Hensgens et al. 1993). Luciferase fused to neomycin phosphotransferase was used to show patterns of expression in transgenic tobacco leaves (Barnes 1990). *β*-glucuronidase has been fused to neomycin phosphotransferase (Datla et al. 1991). *LacZ* fused to *nptII* was used to tag shoot apex-specific genes (Suntio and Teeri 1994). Green fluorescent protein driven by three different constitutive promoters provided a non-invasive way to determine expression in grape as

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controlled by three different promoters (Li et al. 2001). Long-term expression with these fusion genes including a comparison of the fusion gene with a non-fused gene has not been reported.

A problem with *uidA* is that it is frequently silenced. *UidA* silencing is a common occurrence in the progeny of transgenic maize (Zhang et al. 1996), oat (Pawlowski et al. 1998), rice (Kohli et al. 1999), sorghum (Emani et al. 2002), wheat (Stoger et al. 1998), and other monocot plants resulting from biolistics as reviewed (Iyer et al. 2000).

Progressive silencing of transgenes commonly occurs in callus lines making it difficult to use *uidA* as a reporter gene during selection of transformed callus lines. About 60% of the perennial ryegrass callus lines showed a decrease in gus expression after 1 year in culture (van der Maas et al. 1994). Up to 50% of the clonal lines of wheat containing the *npt II* gene were silenced after only 7 months (Muller et al. 1996). Callus lines of *Pennisetum glaucum* transformed with both the *uidA* and *hph* genes showed that 50% of the lines expressed gus initially, and this level decreased with time in culture (Lambe et al. 1995). In all three cases the silencing was attributed to methylation. Differences in long-term expression between *hph* and *uidA* correlated with the methylation that occurred with each gene as *uidA* was silenced more frequently and methylated more often than the *hph* gene (Lambe et al. 1995). Both transgenic rice callus and suspension lines showed decreased gus expression with time in culture (Hensgens et al. 1993; Meijer et al. 1991). In one cell line, silencing of cultured rice cells containing the 35S-*uidA* gene was shown to result from post transcriptional gene silencing (Kanno et al. 2000).

There are different causes of transgene silencing. Repeat genes attract methylation (Malagnac et al. 1997; Wang and Waterhouse 2000) and result in both transcriptional and post transcriptional gene silencing (Matzke et al. 1994; Stam et al. 1997). A major cause of transgene silencing appears to be the presence of multiple transgenes allowing for more opportunities of interaction between similar transgene sequences that result in silencing (Kumpatla and Hall 1998). A molecular analysis of inserted genes has shown that sometimes silencing is caused by introduction of a truncated gene insert (Kohli et al. 1999). Loss of the transgene is a rare occurrence and has been reported to occur in transgenic wheat plants (Srivastava et al. 1996; Stoger et al. 1998).

This study reports that gus was expressed at significantly higher levels in callus lines of *Gladiolus* that contained the *bar-uidA* fusion gene as compared to callus lines that contained only the *uidA* gene. The mechanism for *uidA* silencing was examined in two of the callus lines that expressed very low levels of gus, and it appeared that methylation was not involved. A molecular analysis showed that two of the lines that did not express gus lacked the *uidA* gene, and a third non-expressing line contained a truncated *uidA* gene.

Materials and methods

Cell lines

Embryogenic cell lines of *Gladiolus* were transformed with either the *bar-uidA* fusion gene under control of the CaMV 35S promoter (pDM327 received from David McElroy, Maxygen, Inc., Redwood City, CA) or by cobombardment with pDM307 (Cao et al. 1992) that contains the *bar* gene under control of the CaMV 35S promoter and pBCG (McElroy et al. 1991) that contains the *uidA* gene under control of the CaMV 35S promoter. These cell lines were transformed by biolistics as described in Kamo et al. (2000). Selected callus lines were grown as callus on selection medium consisting of MS basal salts medium (Murashige and Skoog 1962) containing 3% (w/v) sucrose and the following in mg l^{-1}: glycine, 1.0; thiamine, 1.0; pyridoxine, 0.5; nicotinic acid, 0.5; 2,4-D, 2.0 (9 μM), phosphinothricin, 6.0 (AgrEvo Co., Somerville, NJ) and solidified with 2 g l^{-1} Phytagel (Sigma Chemical Company, St. Louis, MO). Callus lines were selected because they grew well on MS selection medium containing 6 mg l^{-1} phosphinothricin.

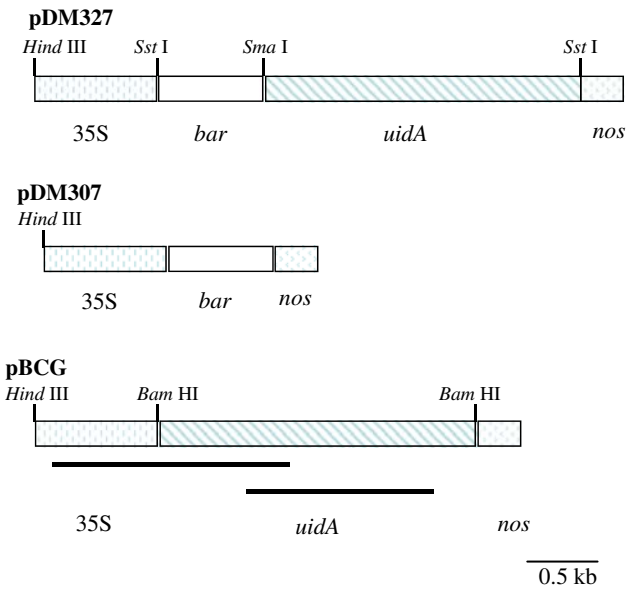

Fig. 1 Diagram of (*top to bottom*) pDM327, the 35S-*bar-uidA* fusion gene, and the two plasmids used for cobombardment: pDM307 consisting of the *bar* gene and pBCG consisting of the *uidA* reporter gene both under control of the 35S promoter. The region of the CaMV 35S promoter and *uidA* gene amplified by the two primer pairs is indicated below pBCG

Twelve lines with the *bar-uidA* fusion gene and fourteen lines resulting from cobombardment were selected for characterization of gus expression during the year that they had been selected and 2 years later. Each of these 26 selected callus lines, approximately 2 g fresh weight, were grown 1 month as a suspension in liquid medium consisting of MS medium with 2.0 mg l^{-1} 2,4-D, and then the cells were plated on solid MS medium with 2.0 mg l^{-1} 2,4-D. Suspensions were cultured on a gyratory shaker at 120 rpm in the dark at 26°C. About 2 months later a single callus was selected and grown as the callus line for future studies. This was done in an effort to prevent chimeras. Callus was transferred monthly to selection medium and grown at 26°C in the dark.

Gus assay

Histochemical staining of cells was performed according to Jefferson et al. (1987). Specific activity of gus expression was measured by fluorimetric determination of methylumbelliferone according to Jefferson et al. (1987). Approximately 300 mg fresh weight of callus was ground in 500 µl extraction buffer (50 mM NaH_2PO_4, pH 7.0, 10 mM EDTA, pH 8.0, 0.1% Triton X-100, 0.1% sarkosyl, 10 mM β-mercaptoethanol) using a mortar and pestle. The ground tissues were sonicated 5 min followed by centrifugation for 5 min at 16,000× *g* in a microcentrifuge. An aliquot of the supernatant was added to the assay buffer (1 mM methyl umbelliferyl-β-D-glucuronide) for incubation at

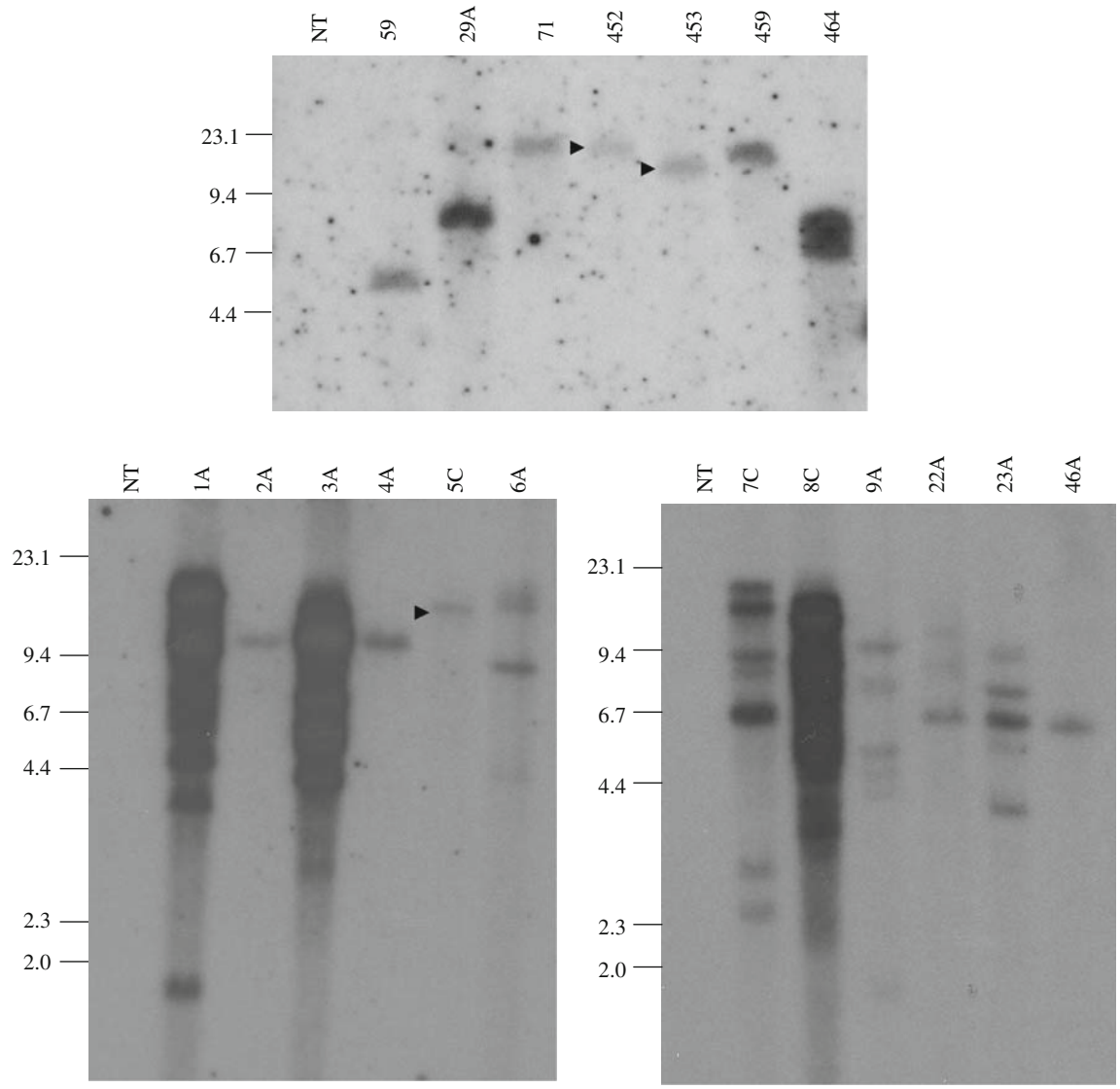

Fig. 2 Southern hybridization of genomic DNA isolated from callus cells of *Gladiolus* probed with the *uidA* gene. Callus had been bombarded with the *bar-uidA* fusion gene. Genomic DNA was digested with *Hind* III, and each *lane* contains 20–30 µg of DNA. The callus *line numbers* are written above each *lane*, and non-transformed (NT) DNA was included as the negative control. Molecular weight markers shown on the *left* are in kb

37°C. Aliquots of the assay buffer with sample were added to 0.2 M sodium carbonate after 0, 15, 30, and 60 min of incubation. Fluorescence was measured with a BioRad VersaFluor Fluorometer set at 360/40 nm for excitation and 460/10 nm for emission. Protein concentration of the tissue extracts was measured using the bicinchoninic (BCA) protein assay reagent (Pierce, Rockford, IL) according to the manufacturer's instructions. The specific activity of β-glucuronidase was measured for three pieces of callus for each cell line.

PCR analysis

The majority of the CaMV 35S promoter and a 5′ region of the *uidA* gene (Fig. 1) was amplified using the forward primer 5′-CTACAAATGCCATCATTGCG-3′ and reverse primer 5′-TAACCTTCACCC GGTTGCCAG AGG-3′ resulting in a 917 bp band. The *uidA* gene was amplified using the forward primer 5′-CTTTAACTATGCCGGAAT CCATCG-3′ and reverse primer 5′-ATTATGCCTTGCGA GGTCGC-3′ resulting in a 1.2 kb band (Fig. 1). The MJ

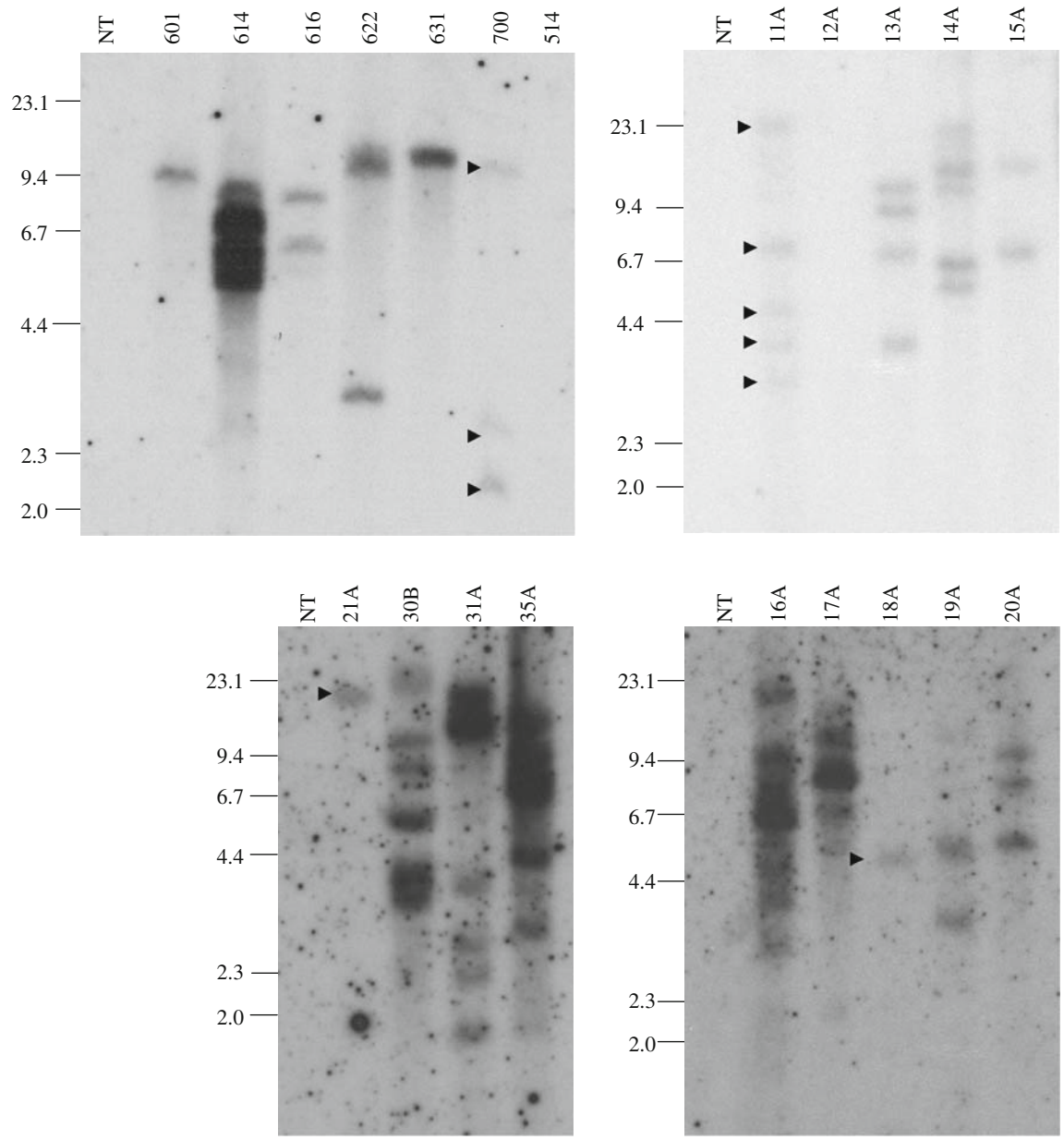

Fig. 3 Southern hybridization of genomic DNA isolated from callus lines cobombarded with pBCG and pDM307. Blots were probed with the *uidA* gene. Each *lane* contains 20–30 μg of genomic DNA that had been digested with *Hind* III. The callus *line numbers* are written above each *lane*, and non-transformed (NT) DNA was included as the negative control. Molecular weight markers shown on the *left* are in kb

Research Microcyler PTC-200 was programmed for 94°C for 2 min, 30 cycles (94°C for 1 min, 55°C for 1 min, 72°C for 1 min), 72°C for 10 min.

Southern hybridization

Genomic DNA was isolated from callus according to the method of Dellaporta et al. (1983). DNA (20–30 μg) was digested with *Hind* III and used for electrophoresis on a 0.7% agarose gel in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0) at 75 V and 50 mA for approximately 4 h. The DNA was transferred to a Nytran nylon membrane (Schleicher and Schuell, Keene, NY) by capillary action (Maniatis et al. 1982). The probes used to detect the *uidA* gene during hybridization were either pDM327 digested with *Sma* I and *Sst* I to release the *uidA* gene that was gel purified using the Prepagene kit (Bio-Rad, Richmond, CA) or a 251 bp probe resulting from PCR using the forward primer 5′-CTTTAACTATGCCGGAATCC ATCG-3′ and the reverse primer 5′-TAACCTTCACCC GGTTGCCAGAGG-3′. The *bar* gene was detected using a 470 bp probe resulting from PCR using the forward primer 5′-CATGAGCCCAGAACGAC-3′ and reverse primer 5′-GTCATGCCAGTTCCCGTG-3′. PCR probes were purified using the Qiaquik PCR Purification Kit (Qiagen Inc., Valencia, CA). Probes were labeled by random primer [α-^{32}P]dCTP using the Decaprime Kit (Ambion, Austin, TX). DNA blots were incubated in prehybridization buffer for 2 h at 42°C followed by incubation for 16 h at 60°C in hybridization buffer (Maniatis et al. 1982). Following hybridization the blot was washed for 15 min, each wash, in 2× SSC, 0.2% SDS then 1× SSC, 0.2% SDS, and lastly 0.1× SSC, 0.2% SDS at 26°C. The blot was exposed at −70°C for 3–5 days with an intensifying screen.

Methylation analysis

Callus lines 616 and 700 were cultured for 45 days on solidified MS basal salts medium supplemented with 20 μM 5-azacytidine, 9 μM 2,4-D, and 6 mg l^{-1} phosphinothricin before the cells were used for histochemical gus assay. Suspension cells of lines 616 and 700 were established, and suspension cells were cultured in either 0, 10, 20, or 30 μM 5-azacytidine and 9 μM 2,4-D. About 1 ml settled cell volume was used for histochemical gus staining after one and 2 weeks of culture.

Genomic DNA was isolated, digested with either *Hpa* II or *Msp* I, and blotted as described above. The DNA blot was hybridized with the *uidA* gene probe labeled as described for Southern blot hybridization.

Northern blot hybridization

RNA was isolated according to the method of Vervoerd et al. (1989) using lithium chloride. RNA was used for electrophoresis using a MOPS/formaldehyde gel followed by transfer of the RNA to a Nytran membrane using capillary movement. RNA blots were incubated in Ultrahyb

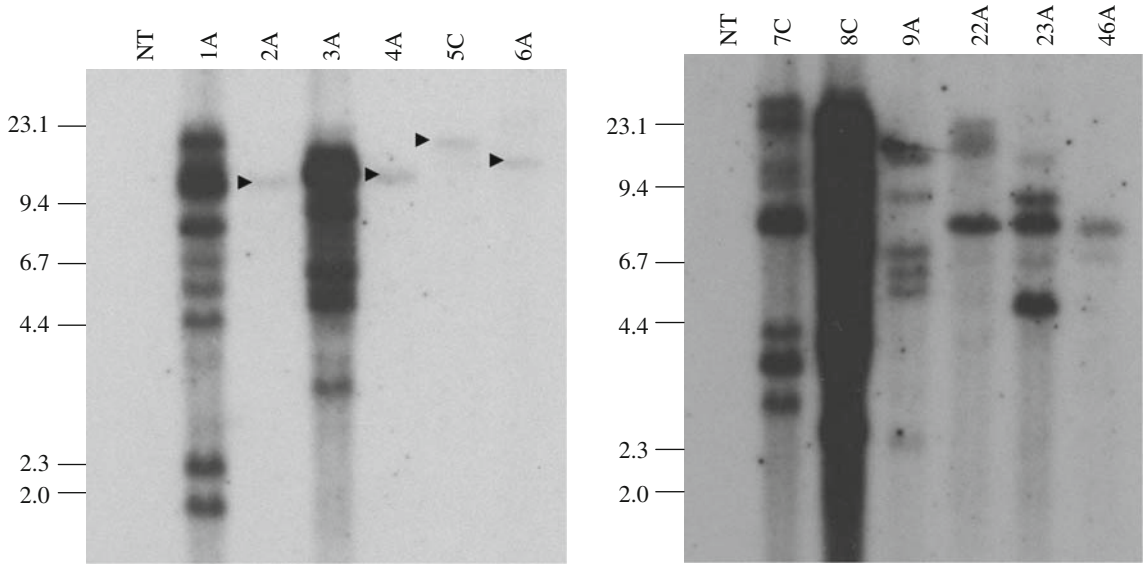

Fig. 4 Southern hybridization of genomic DNA isolated from callus lines that had been bombarded with the *bar-uidA* fusion gene. Blots were probed with the *bar* gene. Each *lane* contains 20–30 μg of genomic DNA that had been digested with *Hind* III. The callus *line numbers* are written above each *lane*, and non-transformed (NT) DNA was included as the negative control. Molecular weight markers shown on the *left* are in kb

(Ambion) for 1 h at 42°C followed by incubation in the same buffer with labeled probe for 16 h at 42°C. The probe was the same *uidA* gene as used for Southern hybridization and labeled with [α-^{32}P] dCTP. RNA blots were washed for 15 min each wash in 2× SSC, 0.2% SDS, then 1× SSC, 0.2% SDS, and lastly in 0.1× SSC, 0.2% SDS at 42°C. The blot was exposed to X-ray film with an intensifying screen at −70°C for 1–5 days.

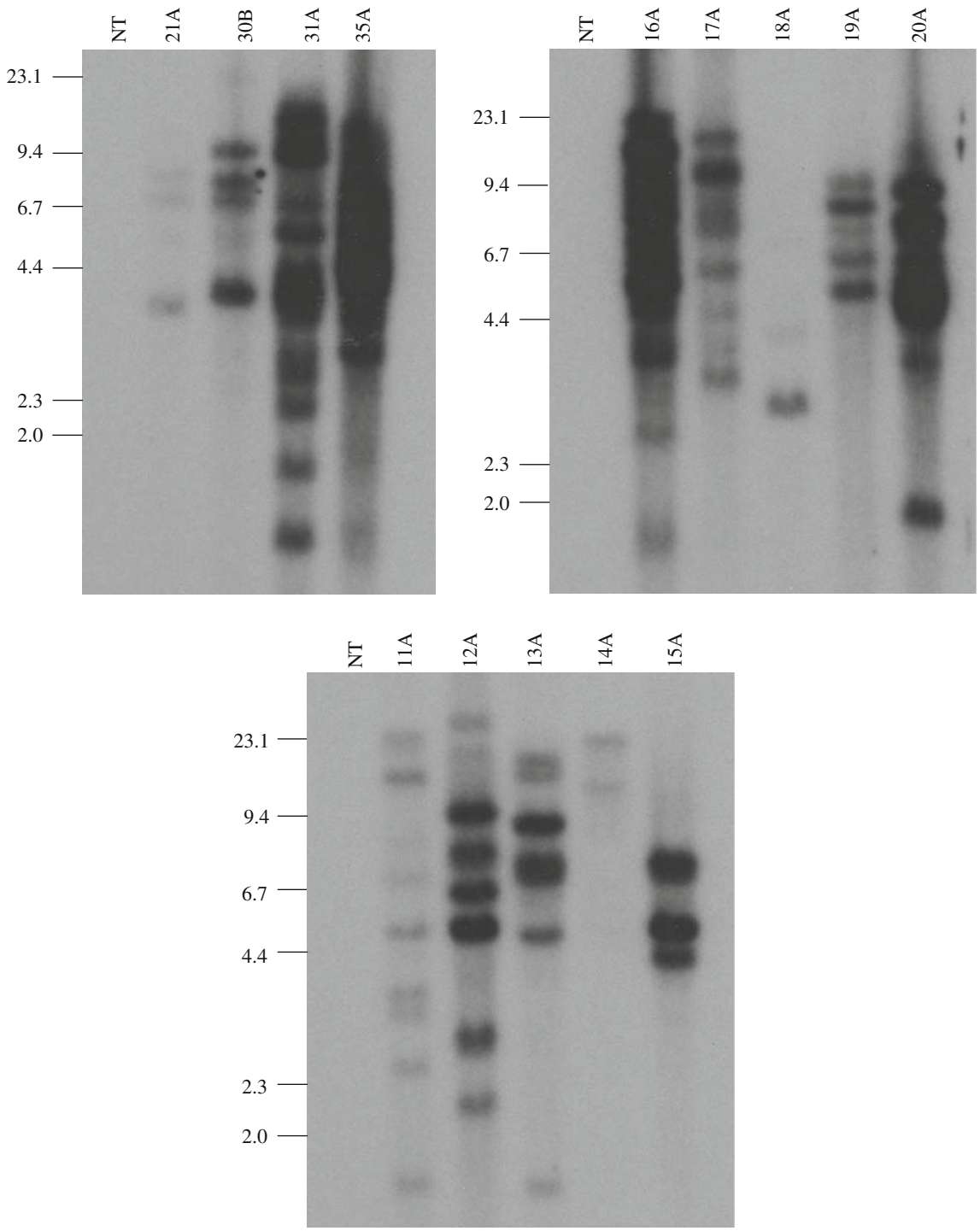

Fig. 5 Southern hybridization of genomic DNA from *Gladiolus* callus lines that had been cobombarded with pBCG and pDM307. DNA blots were hybridized with the *bar* probe. Each *lane* contains 20–30 μg of genomic DNA that had been digested with *Hind* III. The callus *line numbers* are written above each *lane*, and non-transformed (NT) DNA was the negative control. Molecular weight markers shown on the *left* at in kb

Statistical analysis

The specific activity of β-glucuronidase was determined for three pieces of callus for each cell line grown in culture in 2006 and 2008. The natural log of the specific activity was analyzed with the PROC TTest and the Analysis of Variance determined (SAS/STAT 2008).

Results and discussion

Southern hybridization

Genomic DNA from all callus lines was digested with *Hind* III that cuts once just before the CaMV 35S promoter to demonstrate integration of the *uidA* gene and allowing a

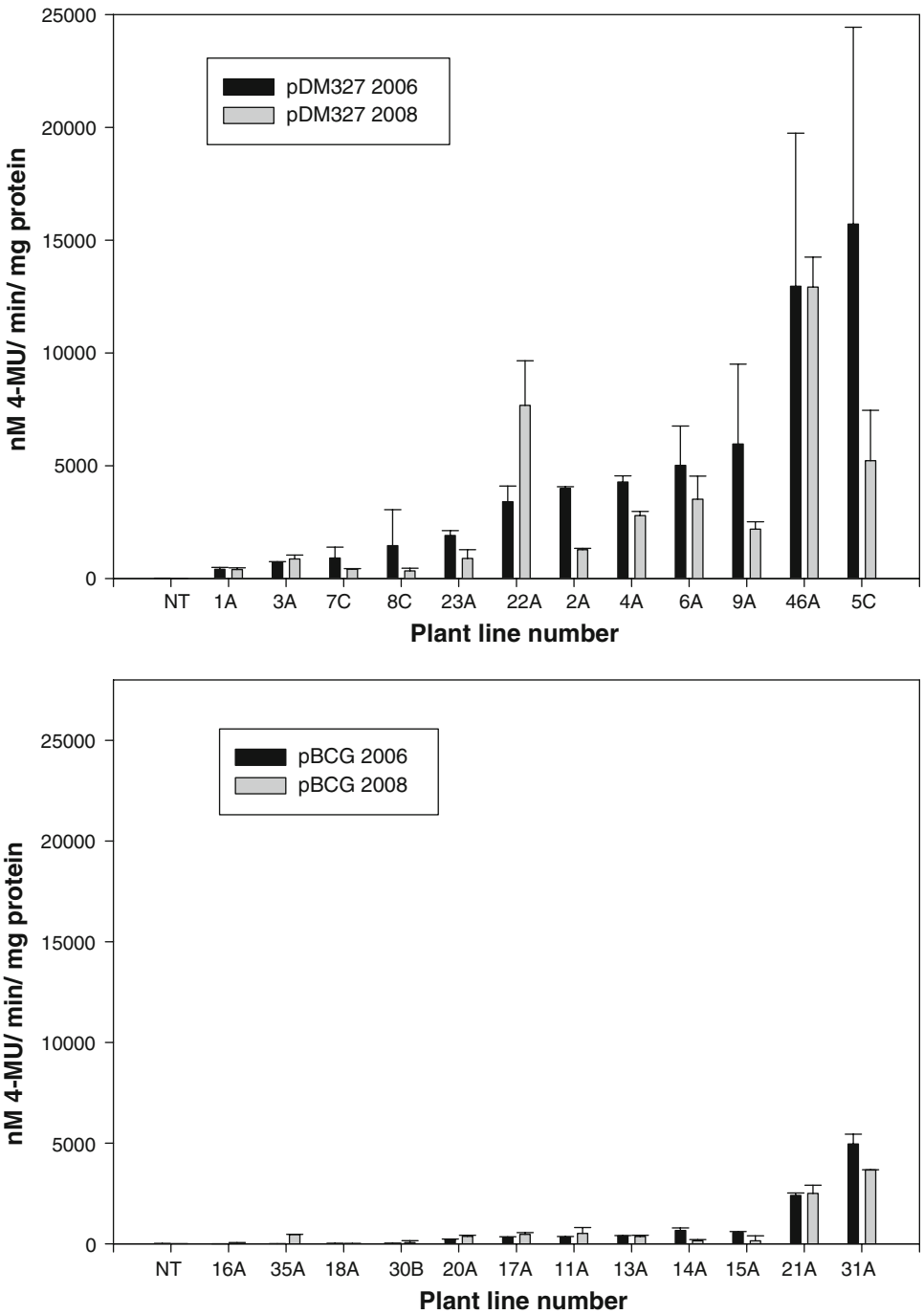

Fig. 6 Specific activity of gus expression for lines of callus containing either the *bar-uidA* fusion gene, pDM327, or the *uidA* gene, pBCG that showed gus expression during either 2006 or 2008. Standard error is indicated for the three callus samples analyzed for each callus line

determination of copy number of the *uidA* transgene (Figs. 1, 2, and 3). All nineteen callus lines bombarded with the *bar-uidA* fusion gene contained the *uidA* gene whereas two of the lines (12A and 514) resulting from cobombardment lacked the *uidA* gene. Integration of the *bar* gene was demonstrated in all lines bombarded with the *bar-uidA* fusion gene and all lines cobombarded with pDM307 (Figs. 4 and 5).

Gus expression

Callus lines with the *bar-uidA* fusion gene were cultured 2 years on MS medium with 6 mg l^{-1} PPT and showed 8.9× higher levels of gus expression than callus lines that had been cobombarded and contained the *uidA* gene of pBCG. The mean levels of specific gus activity were 1,882 nmol/min/mg protein ± 705 (standard error) and 16,834 nmol/min/mg protein ± 5,623 for pBCG and pDM327, respectively. This level of expression was significantly higher following a comparison using the *t*-test analysis (data not shown). The specific activity of gus expression for lines with the *bar-uidA* fusion gene ranged from 324 to 81,650 nmol/min/mg protein, and the range for expressing lines with pBCG ranged from 53 to 9,800 nmol/min/mg protein. The increased level of GUS enzyme activity resulting from transient transformation using a 35S-*bar-uidA* fusion gene rather than the 35S-*uidA* gene occurred for both wheat and maize but not tobacco (Kamo et al. 2000). Results from transformed callus lines of *Gladiolus* were similar to those for cereal monocots because there were higher levels of gus expression in *Gladiolus* using the 35S-bar-*uidA* fusion gene rather than the 35S-*uidA* gene construct. Possibly the *bar-uidA* fusion may stabilize the *uidA* RNA transcript resulting in decreased degradation and consequently higher levels of gus expression than when the *uidA* gene alone is used for transformation.

The levels of gus expression was significantly higher the first year in culture for 12 callus lines with the *bar-uidA* fusion gene as compared to 2 years later (Fig. 6). Others have found a progressive decrease in gus expression as the length of time cells are grown in culture, and this occurred with *Gladiolus* (van der Maas et al. 1994; Lambe et al. 1995; Muller et al. 1996).

All callus lines transformed with the *bar-uidA* fusion gene expressed gus as compared to 15 (71%) of the lines that had been cobombarded. The five callus lines, 514, 616, 700, 19A, and 35A, that either no longer showed detectable GUS activity were further examined to determine the reason for their lack of gus expression. Two of the cobombarded lines, 12A and 514, lacked the *uidA* gene, and line 19A that contained a truncated *uidA* gene did not express gus (Fig. 7). Line 35A that did not express gus contained

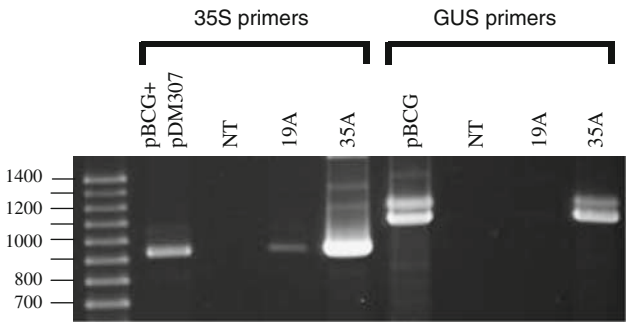

Fig. 7 Callus lines 19A and 35A that did not express gus contained the majority of the CaMV 35S promoter and approximately half of the 5′ end of the *uidA* gene as indicated by PCR analysis using 35S primers. *Line 19A* did not contain the 3′ region of the *uidA* gene as did *line 35A* following amplification with GUS primers

the majority of the CaMV 35S promoter and *uidA* gene as shown by PCR analysis (Fig. 7) so the reason for its lack of gus expression is unknown. GUS was not expressed by line 35A that had been maintained on MS medium with phosphinothricin or medium lacking it indicating that selection for cells with the *bar* gene did not influence gus expression.

Callus lines, 616 and 700, that contained the *uidA* gene of pBCG did not express detectable levels of gus by enzyme analysis. Only a few cells amongst a plateful of cells showed light blue gus staining following histochemical staining. There were 98 and 2 blue spots observed for callus lines 616 and 700, respectively, for one ml of settled cell volume of cells. Northern blot hybridization confirmed that a *uidA* RNA transcript was not detectable in cells of lines 616 and 700 (Fig. 8). Genomic DNA was digested with either *Hpa* II or *Msp* I for detection of methylation within the *uidA* gene. Weber et al. (1990) have reported eight sites that are recognized by *Hpa* II within the *uidA* gene. Both callus lines 616 and 700 showed the same Southern blot hybridization patterns when the genomic DNA was digested with either *Hpa* II or *Msp* I indicating

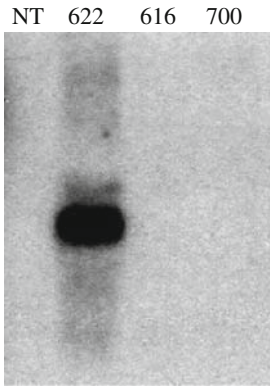

Fig. 8 Northern hybridization using RNA isolated from callus lines 622, 616, and 700 (10 μg/lane) and hybridized with the *uidA* gene

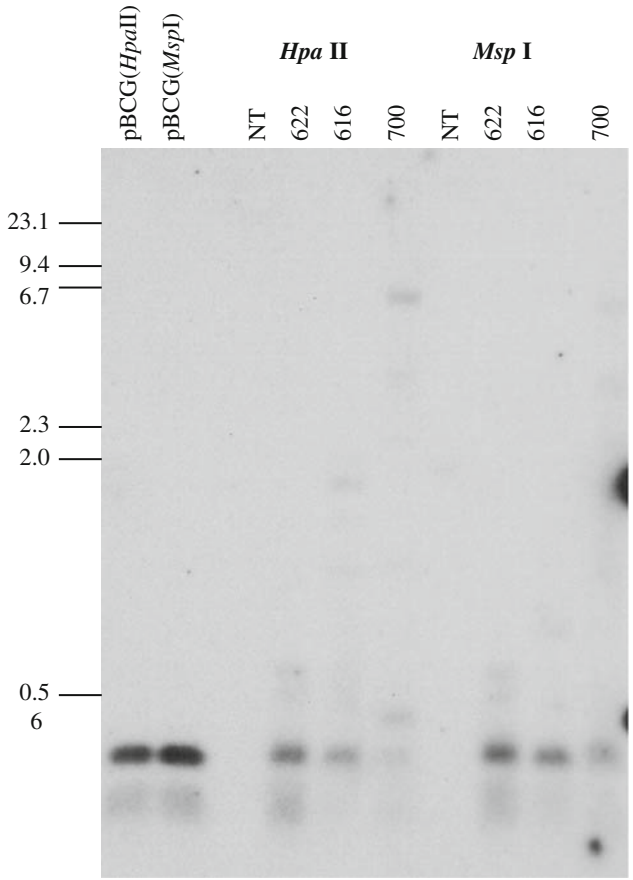

Fig. 9 Each *lane* contains 20 μg of genomic DNA digested with either *Hpa* II or *Msp* I for detection of methylation. The DNA blot was probed with the *uidA* gene. The molecular weight markers shown on the *left* are in kb

that methylation did not appear to occur within the *uidA* gene at those sites (Fig. 9). The possibility that methylation might be occurring at other locations within the *uidA* gene or its promoter was tested by incubating the cells in 5-azacytidine to reverse any possible methylation. Suspension cells of lines 616 and 700 were incubated with 10, 20, and 30 μM 5-azacytidine for 2 weeks, and there was no change in gus expression indicating that methylation did not appear to be the cause of the silenced gus expression in most of the cells for callus lines 616 and 700. In some plant species such as barley (Zhang et al. 1999), methylation occurs when plants are regenerated in vitro, but *Gladiolus* DNA does not appear to be readily methylated.

In conclusion, it was demonstrated that the levels of gus expression are 8.9× higher using the *bar-uidA* fusion gene rather than the 35S-*uidA* gene cobombarded with the 35S-*bar* gene. Currently we are using the *bar-uidA* fusion gene for working out the transformation of other floral monocots that show frequent silencing of the *uidA* gene. *Gladiolus* appears to be a good candidate for genetic engineering because transgene silencing does not occur frequently. Previously twenty-three *Gladiolus* plants containing the *bar-uidA* fusion gene were analyzed after three seasons of dormancy, and all 23 plants continued to express gus (Kamo 2003). In this study only one line, 35A, was silenced out of 37 callus lines that contained the *uidA* gene.

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